EXHIBIT E

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Growth, morphology, and serial transplantation of anaplastic human gliomas in athymic mice

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Abstract

Sixteen of 17 anaplastic human gliomas (AHGs) transplanted into athymic mice produced progressively growing subcutaneous nodules at the site of implantation. Thirty-four of 68 animals (50%) receiving transplanted tissue developed 500 m^3 tumors in 24 to 364 days. Fourteen AHG were passed to a second animal generation, and 11 showed continued growth. Eight of these were serially passed, with one reaching a sixth animal generation, four reaching a fifth, and a three third. Once growth occurred in a second animal generation, no AHGs were lost in subsequent passages because of failure to grow. Of 234 animals receiving tumors beyond the first animal generation, 189 (80.8%) developed tumors. Average doubling times of the exponentially growing tumors in serial passage ranged from 3.0 to 19.1 days. This growth rate tended to increase and stabilize in early animal passages. The tumors growing in animals contained cell types which were present in the original human tumors, including fibrillary and protoplasmic astrocytes, small anaplastic cells, gemistocytes, anaplastic spindle cells, and multinucleate giant cells. Glial fibrillary acidic protein (GFAP) was detected in 15 of 17 biopsy specimens and in 12 of 14 AHGs in animals. These data illustrate the value of the athymic mouse system for the investigation of human brain tumors by demonstrating a high rate of successful transplantation, quantitative growth data on serially passed tumors, and morphological and immunochemical resemblance of the tumors in mice to the original human tumors.

Introduction

The athymic mouse has proven to be a reliable host for the growth of some malignant human tumors and offers the prospect of characterization of human neoplastic cells in an in vivo setting (1). The value of this system would be strengthened by a high rate of successful transplantation and by the maintenance of representative characteristics of individual tumors. This is particularly relevant for anaplastic gliomas, a biologically heterogeneous group of tumors in which experimental data have been derived largely from diverse animal models (2, 3). We have achieved a high rate of successful subcutaneous transplantation of a series of AHGs into athymic mice, and we have evaluated the morphology of the tumors, their expression of a biochemical differentiation marker of astrocytes, and their growth patterns in serial transplantation.

Materials and methods

Animals

Animals were adult athymic BALB/c mice bred at Duke University from an original stock obtained from Sprague-Dawley (Madison, Wisconsin). The animals were maintained under pathogen-free conditions, using sterilized plastic cages covered with polyester bacterial filter tops and were fed sterilized, fat-enriched food with water adjusted to pH 2.3–3.0.

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Tumor transplantation

Tumor specimens were obtained directly from the operating room from patients undergoing resections of malignant brain tumors. A representative sample of tumor was fixed in 10% buffered formalin for histological analysis. The remaining tumor was weighed, mechanically minced, and injected subcutaneously into the right flank of the animals in a volume of less than 1.0 ml using a 16-gauge needle. All procedures were performed under sterile conditions.

Morphology of human tumors

All hematoxylin- and eosin- stained (H&E) slides from each patient's surgical procedure were coded, reviewed, and classified according to the World Health Organization classification for brain tumors (4) by one of us (SHB). Selected sections were also stained with Masson trichrome and Wilder reticulin stains.

Tumor growth and passage

Tumors were measured once or twice weekly with calipers, and the volumes were calculated according to the formula $a^2 \times b/2$, where a = width and b = length (5, 6). For tumor passage, the animals were killed by cervical dislocation, tumors were removed under sterile conditions, and, after appropriate material was obtained for histological analysis, the tumors were passed in a modified tissue press through 30/40 mesh cytosieves. Volumes of 50–200 μd of this processed tissue were then implanted into the right flank of recipient animals using a 20-gauge needle and a Hamilton syringe.

Serially passed tumors were followed until a volume of at least 1 000 mm^3 was achieved. Volume doubling times were calculated from sequential measurements once exponential growth began. A volume of 500 mm^3 was taken as a measure of successful growth as volumes fluctuated somewhat below this level, progressive growth always occurred once this volume was achieved, and 500 mm^3 was on the linear portion of the growth curve in all instances.

Morphology of tumors growing in mice

Portions of subcutaneous lesions from all mice that died spontaneously or were killed were fixed for at least 48 hr in 10% buffered formalin, paraffin embedded, and 5–7 μ sections were stained with H&E. These slides were coded and evaluated for the presence or absence of neoplastic cells. The predominant cellular morphology seen in each lesion was recorded. Stellate and bipolar cells with slender cytoplasmic processes which resembled cell types seen in human astrocytic neoplasms were termed 'fibrillary astrocytes.' Polygonal cells with round nuclei and pink or clear cytoplasm were termed 'protoplasmic astrocytes.' Plump, round cells with abundant eosinophilic cytoplasm were termed 'gemistocytes.' Small round or fusiform cells with scant cytoplasm were termed 'small anaplastic cells.' Large, densely packed, spindle-shaped cells were called 'anaplastic spindle cells.' Large, bizarre multinucleate cells were considered 'multinucleated giant cells.'

Immunohistochemical staining of tumor tissue for GFAP

The tumors were stained for GFAP (7, 8) by the peroxidase anti-peroxidase technique (9) using rabbit anti-human GFAP serum kindly supplied by Dr. Lawrence F. Eng. This technique was applied to formalin-fixed, paraffin-embedded tissue as described in detail by Jones et al. (10). The slides were considered GFAP positive when cells judged neoplastic on morphological grounds contained reaction product.

Results

Morphology of the original human biopsies

Fifteen of the 17 biopsies possessed the typical features of glioblastoma multiforme (GBM). They contained various combinations of fibrillary, protoplasmic, and gemistocytic astrocytes, round or fusiform anaplastic cells, and bizarre multinucleate giant cells (Fig. 1A). Necrosis with or without pseudopalisading, increased vasculature, endothelial proliferation, and mitotic figures were common. One of 17 cases (N-137) was composed of

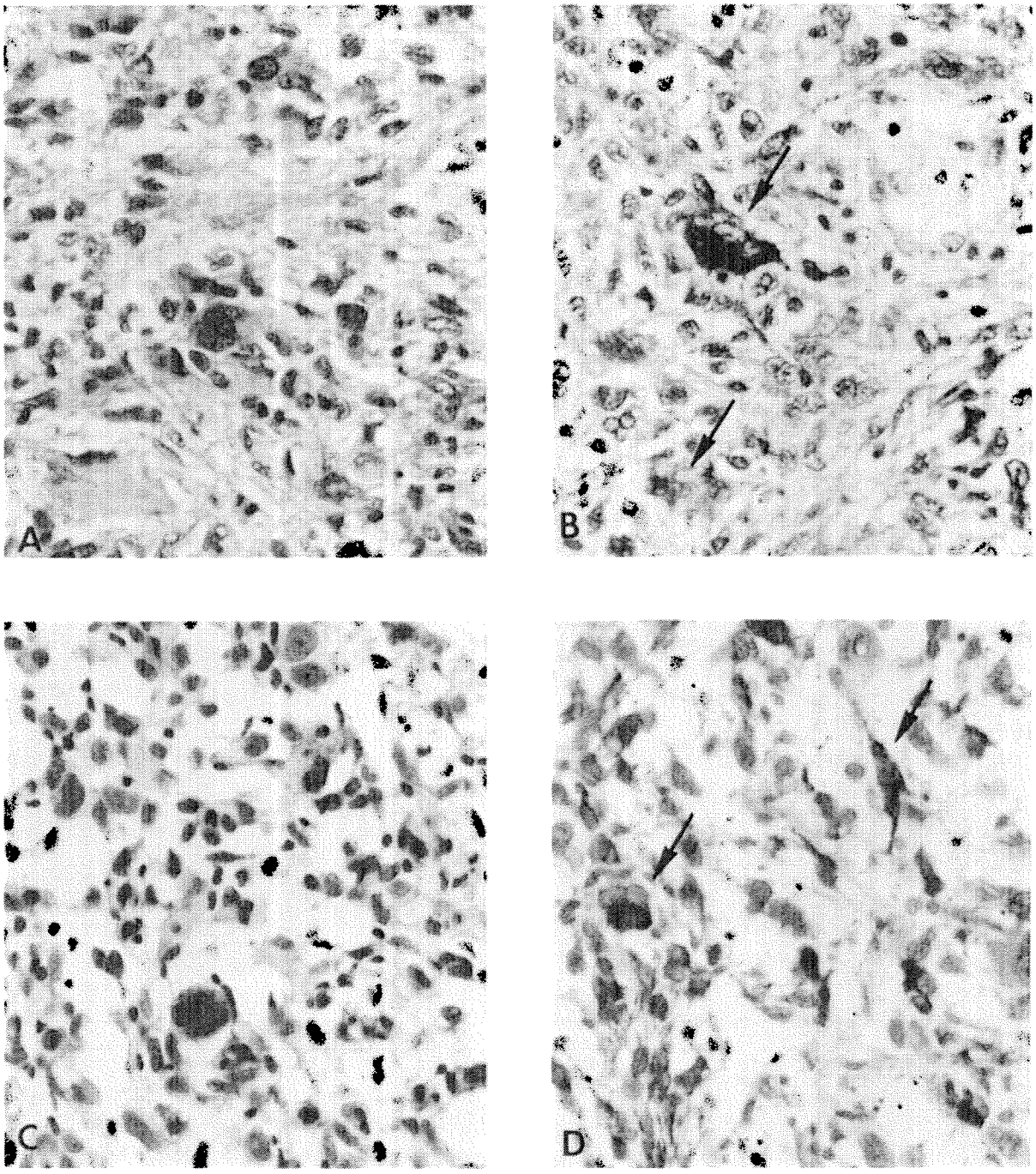

Fig. 1. (A) The original human biopsy in case N-391 is composed of anaplastic fibrillary and protoplasmic astrocytes, with numerous bizarre multinucleate giant cells (H&E × 400); (B) This same field stained with an immunoperoxidase procedure for demonstrating glial fibrillary acidic protein (GFAP) shows cytoplasmic staining (arrows) in a variety of cellular configurations (GFAP × 400); (C) The first passage of N-391 tumor in the mouse shows a mixture of cell types (H&E × 400); (D) Numerous cells are positive for GFAP (arrows) in the first passage tumor in the mouse (GFAP × 400).

Table 1. Growth of 17 anaplastic human gliomas after initial transplantation into athymic mice.

Tumor	No. of animal tumors reaching 500 mm³ No. of animals receiving tumors	Amount of tumor transplanted per animal (mg)	(Median, Range)	
			Days from transplantation to 500 mm³ tumor volume	Tumor volume doubling time (days)
N-90/94	3/12	200	161, 157–277	10.7, 5.7–18.8
N-112/115	8/12	190	139, 65–225	14.5, 8.2–22.0
N-120	3/3	260	219, 156–253	17.8, 6.1–30.8
N-132	5/5	80	108, 99–123	20.6, 8.6–27.8
N-137	0/3*	85	–	–
N-142	0/2**	460	–	–
N-155	0/2***	–	–	–
N-175	0/5+	–	–	–
N-183	3/4	–	327, 320–362	18.1, 12.7–18.1
N-241	4/4	–	119, 112–131	6.9, 5.0– 7.7
N-292/294	2/8	–	170, 104–236	7.0 4.0–10.1
N-338	1/2	–	89	30.1
N-341	0/1++	–	–	–
N-350	1/1	–	43	7.8
N-391	2/2	–	42, 24–60	11.4, 9.1–13.6
N-456	1/1	2 900	63	9.8
N-457	1/1	1 300	0+++	12.5

NA Not available.

* One of these three tumors had reached 88 mm³ on day 156 and was passed to a second animal generation.

** One of these two tumors had reached 418 mm³ on day 265 and was passed to a second animal generation.

*** One of these two tumors had reached 196 mm³ on day 415 when the animal was killed.

\+ One of these five tumors had reached 405 mm³ on day 76 and was passed to a second animal generation.

++ This tumor was treated with 0.125% trypsin for 10 min prior to implantation into the animal. No tumor was evident 165 days after transplantation when the animal was killed.

+++ Smallest volume = 642 mm³ on day 42.

anaplastic astrocytes, but lacked endothelial proliferation, necrosis, and mitotic activity, and it was considered and anaplastic astrocytoma. One case (N-175) was a re-excision of a gliosarcoma. It contained abundant necrosis and small areas of anaplastic spindle cells consistent with a sarcoma.

Growth after initial transplantation

Tissue from the 17 AHG was transplanted into 68 athymic mice in the initial animal passage (Table 1). Each of four AHGs was transplanted into single animals, and 13 were divided into equal portions and transplanted into two or more animals. The amount transplanted ranged from 80–2 900 mg per animal. As the series progressed, we transplanted larger amounts of tumor into fewer individual animals. Two specimens (N-175 and N-457) were obtained from patients undergoing second resections after both radiation and chemotherapy had been administered; in one of these cases (N-457), tissue had also been obtained from the patient's initial resection (N-350).

Sixteen of 17 AHGs (94%) showed progressive growth in the initial passage in at least one of the recipient animals. Twelve produced at least one subcutaneous tumor of greater than 500 m³. Three (N-137, N-142, and N-175) formed small nodules which were passed before reaching 500 mm³. One (N-155) grew in one animal, but the animal was killed because of illness when the tumor was 196 mm³. One AHG (N-341) had shown no evidence of growth when the animal was killed 165 days after transplantation. This tumor was the only specimen exposed to trypsin prior to transplantation. Tumors grew as circumscribed, lobulated masses which sometimes invaded surrounding soft-tissue structures, but the body cavities were not invaded and no metastases were observed. They characteristically regressed for variable periods after transplanta-

Table 2. Morphological and biochemical characterization of anaplastic human gliomas transplanted into athymic mice.

	Human tumor		Animal tumor	
	Histologic diagnosis	GFAP	Predominant cell type	GFAP
N-90/94	Glioblastoma multiforme (GBM)	+	Small anaplastic cells	+
N-112/115*	GBM	+	Fibrillary astrocytes	+
N-120	GBM	+	Protoplasmic astrocytes	ND
N-132	GBM	+	Anaplastic spindle cells	+
N-137	Anaplastic astrocytoma	+	Fibrillary astrocytes**	ND
N-142	GBM	+	Fibrillary astrocytes	+
N-155	GBM	+	Fibrillary and protoplasmic astrocytes	-
N-175	Gliosarcoma	-	Anaplastic spindle cells	-
N-183	GBM	+	Fibrillary astrocytes Gemistocytes	+
N-241	GBM	+	Small anaplastic fusiform cells	+
N-292/294	GBM	+	Fibrillary astrocytes Gemistocytes Small anaplastic cells	+
N-338	GBM	+	Fibrillary astrocytes	+
N-341	GBM	+	NA	NA
N-350	GBM	+	Fibrillary astrocytes**	+
N-391	GBM	+	Fibrillary astrocytes Small anaplastic cells Multinucleate giant cells	+
N-456	GBM	+	Fibrillary astrocytes	+
N-457	GBM	UI	Fibrillary astrocytes	+

NA Not applicable.
ND Not done.
UI Uninterpretable due to necrosis.
* In an accompanying paper (10), this tumor was treated as three separate lines (N-112, N-114, N-115) for morphological analysis.
** Nests of tumor in much fibrous tissue.

tion, leaving either no palpable tumor or a small mass which fluctuated slightly in size until exponential growth began.

Thirty-four of the 68 animals (50%) receiving directly transplanted tumors developed tumor nodules of at least 500 mm^3. Latency from transplantation to 500 mm^3 tumor volume ranged from 24 to 364 days in 33 animals (Table 1). In one animal receiving 1 300 mg of tumor (N-457), the smallest tumor volume was 648 mm^3 on day 42, after which progressive tumor growth occurred. Doubling times of these 34 mouse-borne tumors from 12 AHGs ranged from 4.0 to 30.8 days. There was no significant correlation between latency to 500 mm^3 volume and subsequent doubling time ($r = 0.23$, $p = 0.19$).

Morphology of first passage tumors in mice

The predominant cellular morphology of tumors growing in the initial transplant generation is shown in Table 2. Nine cases showed only fibrillary and protoplasmic astrocytes. Four showed a homogeneous population of round, fusiform, or spindle-shaped anaplastic cells. Three showed a mixture of cell types (Fig. 1C), and no tumor was available for examination in one case.

Growth in serial transplantation

Fourteen of the 16 AHGs which showed progressive growth in the first animal passage were passed into a second generation. Eleven of these 14 showed progressive growth with animal tumors reaching 500 mm^3, while no tumors had appeared in three

Table 3. Growth of serially transplanted anaplastic human gliomas in athymic mice.

Tumor	Animal passage level	No. of animal tumors reaching 500 m^3/ No. of animals receiving tumor	Mean tumor volume doubling times (Days ± S.D.)
N-90/94	2	4/6	6.4 ± 1.3
	3	14/14	6.1 ± 2.1
	4	4/4	5.7 ± 0.9
	5	4/4	8.7 ± 0.7
N-112/115	2	17/22	15.5 ± 8.3
	3	25/34	8.5 ± 4.3
	4	17/23	7.2 ± 5.0
	5	9/9	5.0 ± 1.1
N-120	2	5/8	8.6 ± 2.4
	3	4/10	8.8 ± 2.9
N-132	2	5/12	15.2 ± 6.2
	3	1/5	8.8
N-175	2	2/2	8/2 ± 0.1
	3	4/4	3/7 ± 0.4
	4	5/5	3.0 ± 1.6
	5	2/2	4.0 ± 0.7
	6	1/1	3.3
N-241	2	4/4	3.6 ± 0.6
	3	5/5	3.2 ± 1.0
	4	11/11	4.0 ± 0.8
	5	4/4	5.0 ± 1.3
N-294	2	1/2	11.4
N-350	2	1/1	9.6
N-391	2	3/3	8.9 ± 0.6
	3	2/2	19.1 ± 10.1
N-456	2	2/2	6.0 ± 1.7
	3	5/5	6.0 ± 1.1
	4	14/16	7.8 ± 2.1
	5	10/10	3.3 ± 0.5
N-457	2	4/4	7.1 ± 1.5

(N-338, N.142, and N-137) when the animals were killed 31, 156, and 274 days after passage. One AHG (N-175) was serially passed to a sixth animal generation, four (N-90/94, N-112/115, N-241, and N-456) reached a fifth generation, three (N-120, N-132, and N-391) reached a third, and three (N-292/294, N-350, and N-457) were not passed beyond a second (Table 3). Tumors were frozen for storage at each animal passage level. Once growth occurred in a second animal generation, no AHGs were lost because of failure to grow in serial transplantation.

Of 234 animals receiving tumor from the second through the sixth passage levels 500 mm^3 tumors were achieved in 189 (80.8%, Table 3). Average tumor volume doubling times ranged from 3.0 days (N-175, fourth passage) to 19.1 days (N-391, third passage). Within individual AHGs there were tendencies for doubling times to decrease (Tables 1 and 3) and growth patterns to stabilize (Fig. 2) in early animal passages.

Morphology of serially passaged mouse tumors

With serial passage, tumors frequently became more densely cellular, but individual cellular morphology remained unchanged in four of five tumors reaching at least the fourth animal passage level. In one case (N-456) a population of anaplastic spindle cells gradually became dominant. The morphological evolution of these tumors in serial animal passage is discussed in greater detail in a companion paper (10).

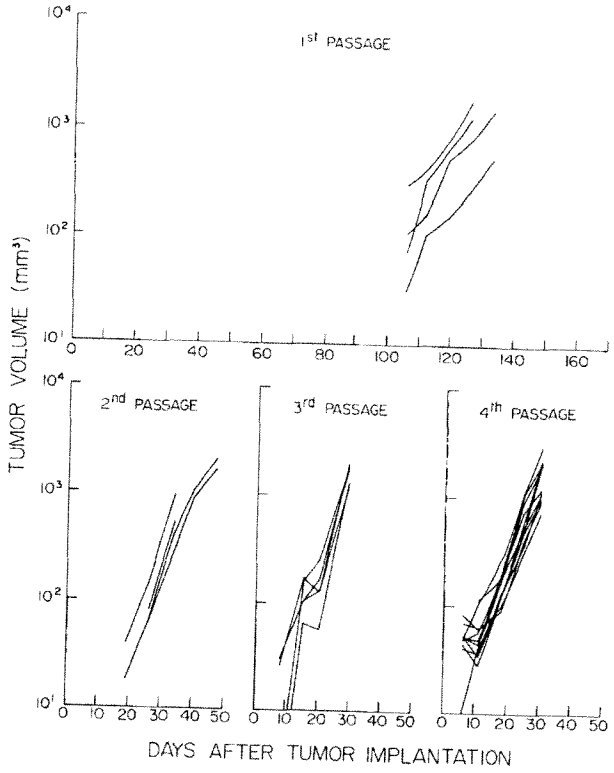

Fig. 2. Graphs illustrate the growth of human tumor N-241 in serial transplantation in athymic mice. In serial passage latency declined and growth patterns stabilized.

GFAP

Fifteen of 17 AHGs contained GFAP in the original biopsy material (Fig. 1*B*, Table 2). Animal tumors in early passage from 14 of these AHGs were also examined, and 12 were positive (Fig. 1*D*). One tumor which was positive in the biopsy was negative in the animal (N-155). The other negative animal tumor was an anaplastic spindle tumor from a patient with gliosarcoma (N-175). Two AHGs which were positive in the biopsy (N-120 and N-137) were not examined in the animal. Changes in the pattern of GFAP expression in serial passage are discussed by Jones et al. (10).

Discussion

Growth of transplanted tumors in athymic mice

The theoretical advantages of the athymic mouse for the investigation of human tumors center on our ability to study proliferating neoplastic cells of human origin in an animal system. However, not all human tumors grow after transplantation into these animals. Melanomas (1, 11), lung cancers (12, 13), and malignant tumors of the gastrointestinal tract (12, 14) have been the most readily established tumors in this system, while tumors of the hematopoetic system and endocrine-dependent tumors have been more difficult to grow (11, 12, 14). In the largest series, from 14.6% (12) to 56% (1) of transplanted tumors have grown in athymic mice, depending primarily on the tumors under consideration, but also on the techniques and definitions utilized. Irradiation of the recipient animals has been reported to increase the rate of successful tumor transplantation (15).

Primary brain tumors have also been reported to grow successfully in these animals. Rana et al. (16), Reid et al. (12), and Wara et al. (17) have reported subcutaneous tumor growth in individual human GBMs transplanted into athymic mice. Shapiro et al. (18) successfully transplanted seven of seven anaplastic brain tumors subcutaneously and six of seven intracerebrally. The two serially transplanted tumors they reported were morphologically both 'gliosarcomas'. In a subsequent report these investigators described 19 successful subcutaneous transplantations of human gliomas, but neither tumor morphology nor growth data were provided in detail and a number of animals were lost due to murine hepatitis (19).

In this series, 16 of 17 (94%) AHGs showed progressive growth after subcutaneous transplantation. The single tumor which did not grow had been exposed to trypsin prior to transplantation, and this may have affected its ability to grow. The high rate of successful 'takes' with AHGs is unexplained; specifically, there are insufficient data to permit conclusions about the relative importance of technical and biological factors. However, the health of our animal colony has permitted a period of observation which exceeds that reported in most series. A number of AHGs requirted over six months before growth was apparent, and in one case (N-183, Table 1) over 300 days elapsed before progressive growth appeared in three of four animals in which tumor had been implanted. These three tumors developed at the site of implantation were grossly similar to the tumors growing in other animals, were cytologically compatible with anaplastic glial cells, and

contained GFAP (Table 2). We presume that a small number of tumor cells survived the transplantation and required a prolonged period of time, even with exponential growth, to become apparent. Other factors, as discussed by Reid et al. (12), also undoubtedly influence the growth of human tumors in immunologically incompetent animals, including the ability of the animal to vascularize the tumor, the hormone-dependency of the tumor, and residual immunologic activity of the recipient animals, but no attempt will be made to analyze those factors here.

Achievement of a tumor volume of 500 mm^3 was considered evidence of successful growth as tumor volumes fluctuated below this level and 500 mm^3 was on the linear portion of the tumor growth curve in every instance. The time required to reach a volume of 500 mm^3 after transplantation was highly variable, ranging from 24 to 362 days in 33 animals from 11 AHGs, possibly because individual animals received larger initial tumor volumes in some cases. The correlation of time to 500 mm^3 tumor volume and subsequent doubling times of the respective progressively growing tumors in the first animal passage was poor, indicating the relatively minor influence of the intrinsic growth rate of these tumors on this variable initial latency period.

Morphology of tumors in mice

The subcutaneous tumors growing in mice contained morphologic cell types which could be recognized in the parent human tumor, but there was a tendency for the mouse-borne tumors to be more homogeneous. Thirteen of 16 tumors in the first animal passage contained a uniform population of either fibrillary and protoplasmic astrocytes or anaplastic cells, while three animal tumors (N-183, N-292/294, and N-391) contained a mixture of cell types. The histologic pattern was not changed in serial passage in four of five tumors passed beyond a third animal generation, although there was a tendency for tumors to become more densely cellular. The remaining tumor became progressively more 'sarcomatous'.

Morphological similarity to the parent tumor has been reported with most tumors successfully transplanted into athymic mice. Povlsen et al. (20) reported a strong correlation between the human and mouse tumors, noting particularly that 'special properties . . . such as mucin production and formation of melanin granules are preserved.'

Giovanella et al. (1) described similar histologic identity in a variety of carcinomas. Ikeuchi et al. (21) found consistent preservation of cytological features of a series of transplanted lung tumors and included one example of a tumor which produced anti-diuretic hormone, adrenocorticotropic hormone, melanocyte-stimulating hormone, and calcitonin in the mouse. Some authors have found that some of the differentiated features of the human tumors have been lost in the animal (14), a change which has been attributed to either 'dedifferentiation' or selection for 'the most poorly differentiated parts of the primary tumor.' The transplanted GBMs reported by Rana et al. (16) retained the cytological features of the original tumor, including pleomorphism, palisading, and necrosis, while Wara et al. (17) reported a GBM which became 'more sarcomatous' with successive animal passage. Reid et al. (12) described a grade IV astrocytoma which became more densely cellular in serial passage in the athymic mouse. The present series of 16 transplanted AHGs includes examples similar to each of these previously reported cases. These differing observations concerning the morphology of human gliomas when grown subcutaneously in nude mice are, therefore, not contradictory but represent variable behavior among the individual tumors.

The presence of glial fibrillary acidic protein (GFAP), a specific marker of fibrillary astrocytes (22), is a further indication of the cellular origin of these mouse-borne tumors. Though cytologic identity between the biopsy material examined for GFAP and the bulk of tissue transplanted into the animal could not be assured, all but one AHG which contained GFAP in the biopsy material was also positive in the animal.

Growth of tumors in serial animal passage

Eleven of 14 AHGs passed into a second animal generation produced at leat one 500 mm^3 tumor. Of the three which did not grow in a second passage, two had been passed from small (< 500 mm^3) first passage tumors which were not clearly established, and one (N-338) was observed in the second passage for only 31 days. No other tumors were lost due to failure to grow in serial passage. Overall 80.8% of

animals receiving tumor in serial passage developed progressive tumor growth, and this figure increased to 96.4% (80/83) in the eight most recent tumors, with increasing refinement of our techniques. While some differences in 'take' were apparent among different tumors (e.g., N-132 vs. N-241), we cannot be sure that these differences were due to properties of the tumors rather than to technical factors. Since tumors were passed into a number of animals simultaneously, any technical error in an individual passage would be magnified.

Growth rates, as determined by tumor volume doubling times, tended to stabilize in serial passage. First and second passage tumors tended both to grow somewhat more slowly and to show greater inter-animal variability. There was also a tendency for some AHGs (e.g., N-175 and N-241) to grow more rapidly and with less variability than others, but overall the number of animals was insufficient to allow conclusions about these differences.

Most investigators studying human tumors in the nude mouse have reported relatively constant tumor growth rates in serial animal passage. Povlsen et al. (23) noted variation in growth rate among different tumors but a 'constant' pattern within individual tumors in serial passage, though quantitative data were not provided. Rae-Venter and Reid (24) also found that breast tumors maintained their growth pattern in serial passage. Mattern et al., on the other hand, reported growth acceleration in serial animal passage in a series of lung tumors (25). While Reid et al. (12) reported that 'the growth rate assumed in the first nude mouse host remains constant throughout subsequent passages,' they noted an exception to this in one anaplastic glioma which showed an increasing growth rate in serial passage, an observation they explained by assuming 'selection . . . for the fastest growing tumorigenic cell type.' Shapiro (18) also noted an increasing growth rate in two serially transplanted gliosarcomas in the early animal passages in a pattern similar to that reported here, although he did not comment on inter-animal variability in serial passage.

Human anaplastic gliomas show striking morphological and biological heterogeneity (26–29). It is not known which of the many cellular components of such a tumor are truly neoplastic or whether the different neoplastic cell populations have different growth potentials. Selection in the animal for the most rapidly growing portion of the transplanted tumor may explain the patterns of growth in serial animal passages reported here and by others. Alternatively, adaptation to an unfamiliar environment might produce behavioral changes in a neoplasm without altering its fundamental characteristics.

The high rate of successful transplantation demonstrated here indicates that this system is particularly appropriate for the analysis of individual human glial neoplasms, including determinations of their therapeutic sensitivities. Bullard et al. (6) have reported the results of treatment with 1-3, bis (2-chloroethyl)-1-nitrosourea (BCNU) of three human glioma-derived permanent cell lines growing in athymic mice, and Shapiro et al. (18) have treated two human glioma xenografts with a battery of agents including BCNU and procarbazine. Use of a variety of chemotherapeutic agents against a series of transplanted, serially passed human gliomas in athymic mice should provide important information about the variability and patterns of chemosensitivity of these tumors.

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